detectable changes in glycolysis that could serve as biomarkers of target suppression in human cancer cells.
Methods: We used the MEK inhibitor CI-1040 and three human cancer cell lines, HCT116 (colorectal), SKMEL-28 and WM 266.4 (melanoma). Cells were treated with 20 μM of CI-1040 for 16 h and 24 h (HCT116) or with 1 μM of CI-1040 for 24 h (SKMEL-28 and WM 266.4). Post-treatment, 10^7-10^8 cells were extracted with dual phase extraction and ^{1}H MRS spectra of the water soluble metabolites were acquired on an 11.7 T Bruker Avance spectrometer. In addition, cell culture media from 16 h treatments with HCT116 cells were analysed.
Levels of P-ERK and downstream targets cyclin D1 and pRB, were evaluated with Western blotting to confirm successful MEK signalling inhibition in response to treatment.
Results: Exposure to CI-1040 led to a marked decrease of ERK phosphorylation as well as levels of cyclin D1 and pRB as shown by Western blotting. Post-treatment, ^{1}H MRS data revealed decreased intracellular lactate levels to 44±10% at 16 h ($n = 3$, $P = 0.01$) and 62±14% at 24 h ($n = 2$) in HCT116 cells. Intracellular lactate levels decreased to 45±18% in SKMEL-28 cells and to 45±2% in WM 266.4 cells at 24 h post-treatment ($n = 2$). Extracellular lactate levels were unchanged 16 h post-treatment in HCT116 cells.
Conclusion: These results demonstrate that MEK inhibition leads to modulation of glycolysis in human colorectal cancer and melanoma cells. Our findings need to be evaluated further in vivo and by the use of additional MEK inhibitors, nevertheless they suggest a role for lactate as a potential non-invasive MRS biomarker of response to MEK targeted therapeutics.
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81 POSTER

Preclinical pharmacodynamic markers of MGCD265, a potent orally active c-Met/VEGFR multitargeted kinase inhibitor in Phase I clinical trials

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Background: MGCD265 is a novel multitargeted receptor tyrosine kinase inhibitor in Phase I clinical trials. MGCD265 inhibits the activation of key regulators of cancer development and progression: the receptor tyrosine kinases c-Met, all three members of the VEGFR family, Tie-2 and Ron. This unique profile distinguishes MGCD265 from other agents that target c-Met or VEGFRs alone. Preclinical evaluations demonstrate that MGCD265 abrogates c-Met and VEGFR-mediated cell-based responses with nanomolar potencies and displays potent anti-tumor activities in multiple human xenograft models. In support of the clinical development of MGCD265, we have analyzed pharmacodynamic markers in tumor tissues and plasma from human xenograft mouse models.
Methods: Pharmacodynamic markers in xenograft tumor tissues were analyzed by immunohistochemistry and western blotting. Plasma markers were assessed by ELISA.
Results: We demonstrate that in correlation with potent tumor growth inhibition, MGCD265 abolishes target enzyme phosphorylation and the activation of downstream signaling pathways in xenograft tumor tissues. These occur with a concomitant reduction in the mitotic index and an increase in the level apoptotic markers. Moreover, it has been suggested that increased tumor malignancy correlates with an increase in the plasma level of proteolytically cleaved c-Met receptor (shed-c-Met). Consistent with this notion, we demonstrate a diminution in the level of circulating c-Met following MGCD265 treatment in mice bearing xenografts, in parallel with tumor shrinkage. Furthermore, the plasma levels of the angiogenic factors, VEGF and HGF, that are regulated by the targets, and that have been linked to poor patient prognosis, are also reduced in MGCD265 treated animals.
Conclusion: Thus, as revealed in preclinical models, monitoring MGCD265-mediated target enzyme inhibition in tumor tissues, and assessing levels of circulating angiogenic factors that are target-regulated, may be used as exploratory markers in Phase I clinical trials of MGCD265.

82 POSTER

Prevalence of G12R or Q61H K-Ras mutations in pancreas cancer and development of Ras-targeted immunotherapy

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Background: Aberrant constitutive signaling by Ras oncoproteins with mutations at codons 12, 13 and 61 drive uncontrolled cell proliferation and tumorigenesis. We have developed targeted immunotherapy to specifically eliminate tumor cells expressing mutated Ras and are genotyping pancreas cancer for the incidence of specific ras point mutations.
GI-4000 tarmogens for immunotherapy with whole, heat-killed yeast expressing mutated Ras proteins were originally a three product series targeting tumors expressing ras codon 12 mutations encoding G12V (GI-4014), G12C (GI-4015) or G12D (GI-4016). Each of the original products also targeted Q61R and Q61L mutations. In preclinical studies, the original GI-4000 tarmogens stimulated mutation-specific ablation of tumor cells when the Ras antigen mutation expressed in yeast matched that found in the tumor. Ras mutation-specific cellular immune responses were shown in 90% of subjects from a Phase 1 study of GI-4000 in colorectal and pancreas cancer.
Experimental procedures: Tissue was obtained by surgical resection of tumors from 124 US and India subjects with Stage I and II pancreas cancer screened for enrollment in a placebo-controlled Phase 2 trial of adjuvant gemcitabine chemotherapy plus immunotherapy with GI-4000 tarmogens. Tumors were genotyped for K-, N- and H-ras DNA sequences by nested PCR amplification including peptide-nucleic acid oligomer clamping, followed by DNA sequencing. The GI-4020 tarmogen was engineered to express G12R- and Q61H-mutated Ras protein. CD8 T cells from mice immunized with GI-4020 or control yeast were mixed with tumors expressing G12R- or Q61H-mutated Ras, then implanted and monitored for control of tumor growth.
Results: Tumor genotyping for the Phase 2 study revealed that 83% of pancreas cancers had K-Ras mutations, predominantly G12V or G12D. However, 19% of these harbored G12R or Q61H mutations, where 5−6% of tumors harbored the Q61H mutation, which was at much greater frequency than previously documented. Preclinical studies demonstrated that administering GI-4020, which targets these mutations, specifically activated T cells against tumors with G12R- or Q61H-mutated Ras.
Conclusions: G12R- or Q61H-mutated Ras is found in pancreas cancer with much higher incidence than previously reported. GI-4020 immunotherapy targets these 2 mutations and is being tested in the ongoing Phase 2 trial. The current four GI-4000 products now cover 92% of mutated Ras-bearing pancreas cancers.

83 POSTER

A robust and quantitative biomarker assay for SB939, a potent, orally-active HDAC inhibitor

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Background: Histone deacetylase inhibitors (HDACi) are therapeutic agents, which induce tumor cell cytostasis, differentiation and apoptosis. SB939 is a pan-HDACi with superior pharmaceutical and pharmacokinetic properties that is currently in Phase I trials. We have developed a sensitive and quantitative Western blot assay for acetylated histone 3 (AcH3) as a pharmacodynamic (PD) readout for the target efficacy of SB939.
Material and Methods: The assay was validated by determining the effects of ex-vivo treatment with SB939 on human tumor cell lines as well as on human peripheral blood mononuclear cells (PBMCs) from healthy donors. In addition, AcH3 was determined in normal as well as tumor tissues after oral treatment of tumor bearing mice with SB939. The doses and schedules used corresponded to those in the ongoing Phase I trials where this assay is currently being employed to assess target efficacy on patient PBMCs.
Results: AcH3 could be detected after ex-vivo treatment of tumor cell lines or PBMCs with SB939 for 24 h. The lowest concentration of SB939 yielding a detectable signal was 60 nM for RAMOS cells and 125 nM (44 ng/ml) for PBMCs. The minimum amount of protein needed to detect AcH3 in cells was 1.56 μg. In the animal studies orally dosed SB939 led to a strong induction of AcH3 only in tumor tissue with no basal level detected in other tissues. In all normal tissues tested, the induction of AcH3 was less than in the tumor tissues indicating selectivity of SB939 for tumor tissue. In the clinical samples assessed, a dose-dependent AcH3 response was detected with the strongest signal observed 3 h post-dose. Comparison of

the biomarker response (PD) with the pharmacokinetic (PK) data showed that the maximum biomaker response occurs after the T_{max}.
Conclusions: A robust, sensitive and quantitative Western blot assay for measuring AcH3 in tissues has been developed to evaluate the target efficacy of SB939 and is currently being used to study PK/PD relationships in the ongoing Phase I clinical trials. Preliminary data show that the excellent PK/PD relationships observed in pre-clinical models are translated to the clinic.

84 POSTER

Development of predictive markers of responsiveness to the MEK 1/2 Inhibitor AZD6244 in Colorectal Cancer (CRC)

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Background: The ERK pathway is constitutively activated in a several types of human cancers including CRC. The MAPK/ERK kinase, MEK, occupies a central role in the ERK pathway and therefore, MEK inhibitors constitute a promising class of novel targeted cancer therapies. AZD6244 (ARRY-142886) is a potent and selective MEK1/2 inhibitor with nanomolar potency against cell lines and activity in several in vivo models of CRC. In a phase I trial of AZD6244, inhibition of ERK phosphorylation was noted in peripheral blood mononuclear cells and tumor biopsies. Despite the enthusiasm for this class of agents in tumors with a high incidence of RAS or BRAF mutation, clinical results indicate that improved patient selection strategies are needed. The goal of this study was to identify predictive markers of sensitivity or resistance to AZD6244 in order to develop a rational basis for patient selection and combination therapy in CRC.
Methods and Results: A panel of 30 CRC cell lines was exposed to varying doses of AZD6244 (0.078 μM-5.0 μM) and analyzed for inhibition of proliferation using the sulforhodamine B (SRB) assay. Cell lines were designated sensitive (S) or resistant (R) based on IC50's less than (S) or greater than (R) 1 μM. Cell lines were assessed for pre-drug and post-drug levels of pERK by immunoblotting, indicating no association between responsiveness to AZD6244 with either baseline or post-treatment activation of the MAPK pathway. Therefore, to identify predictive markers, five S and R cell lines, differing in IC50 by 10-fold, were subjected to gene array analysis. ANOVA comparison of gene expression profiles between R and S cell lines revealed over 100 differentially expressed genes with a global p-value of <0.04, whereas 64 transcripts met the $p < 0.001$ criteria. We selected three genes, FZD2 (frizzled-2), TDGF-1 (teratocarcinoma-derived growth factor) and AKR1C3 (aldo-keto reductase 1C3) based on their relevance to cancer biology and high level of differential expression between S and R cell lines. Next, we silenced FZD2 expression in S and R cell lines using stably transfected shRNAs and examined effects on the S or R phenotype. We initially examined the effects of the FZD2 gene knock-down in the R cell line, SW480 and a nearly complete reduction of FZD2 was achieved, as confirmed by immunoblot. These cell lines were exposed to AZD6244 and assayed by SRB. We observed more than 50% reduction in the IC50 to AZD6244 compared to wild-type and scrambled shRNA controls. These results indicate that FZD2 may be a predictive marker, that when modulated, increases responsiveness to AZD6244.
Conclusions: Potential predictive biomarkers for sensitivity and resistance to AZD6244 were identified through gene array analysis. Specific knock-down of the FZD2 resulted in rendering the resistant cell line SW480 more sensitive to the AZD6244. These biomarkers will be further validated in pre-clinical xenograft models.

85 POSTER

Pharmacodynamic and efficacy relationship of MLN4924, a novel small molecule inhibitor of Nedd8-activating enzyme, in human xenograft tumors grown in immunocompromised mice

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MLN4924 is a first-in-class, small molecule inhibitor of the Nedd8 Activating Enzyme (NAE). We investigated whether the mechanism of action of MLN4924 identified in cultured cells is also detectable in tumor xenografts from mice treated with MLN4924, and how the pharmacodynamic effects of MLN4924 in these tumors correlate with efficacy.
Inhibition of NAE leads to decreased neddylation and thereby decreased activity of the cullin-dependent ubiquitin ligases (CDLs), enzyme complexes which control the ubiquitination and subsequent degradation of proteins with important roles in cell cycle progression. In cultured cells, NAE inhibition with MLN4924 resulted in the elevation of multiple substrates of the CDLs, an accumulation of cells with increased DNA content (>4N), a DNA damage response, and induction of cell death. This phenotype (increased DNA content, DNA damage response and cell death) is similar to that induced by over-expression of Cdt-1, a critical DNA replication licensing factor which is a substrate of CDLs.
In vivo administration of MLN4924 at well-tolerated doses to mice harboring subcutaneous human tumor xenografts resulted in a dose-dependent pharmacodynamic (PD) response of Nedd8 pathway inhibition in tumors derived from lung, breast, and colon carcinoma as well as lymphoma. A single dose of MLN4924 caused inhibition of neddylated cullins and stabilization of the CDL substrate Cdt-1 in all models tested. We further explored the PD/efficacy relationship in the HCT-116 xenograft model, which showed tumor growth inhibition in response to MLN4924. A single dose of MLN4924 to HCT-116 xenograft-bearing mice resulted in decreased levels of neddylated cullins, elevation of the CDL substrates Nrf-2, cyclin D1, and Cdt-1, and the phosphorylation of Chk-1 on serine 317, an indication of ATM/ATR activation and a DNA damage response. Multiple doses of MLN4924 on a twice-daily schedule resulted in the sustained inhibition of neddylated cullins, elevation of CDL substrates, and a continued DNA damage response in the xenograft tumors, accompanied by an increase in cleaved caspase-3 levels. Histologic analysis of tumors exposed to multiple doses of MLN4924 revealed the appearance of cells with enlarged nuclei, suggesting an increase in DNA content, and an increase in the number of apoptotic cells compared to untreated xenografts. These results demonstrate that MLN4924 effectively inhibits NAE and that the resulting stabilization of CDL substrates including Cdt-1 is sufficient to activate DNA damage and apoptotic responses in HCT-116 xenograft tumors. PD markers used in the xenograft tumor experiments are being adapted for use in Phase I trials of MLN4924 to support clinical development.

86 POSTER

Aberrant promoter hypermethylation of DAPK gene is an independent prognostic factor in patients with diffuse large B-cell lymphomas

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Background: Diffuse large B-cell lymphoma (DLBCL) exhibits heterogeneous clinical features and a marked variable response to treatment. The aim of the study was to assess the prognostic significance of the methylation status of several tumor suppressor and related genes in DLBCL.
Materials and Methods: We investigated the methylation status of DAPK, GSTP1, P14, P15, P16, P33, RB1, SHP1, CDH1, APC, BLU, VHL, TIMP3, and RASSF1A by methylation-specific polymerase chain reaction in 46 DLBCL specimens from Tunisian patients with well known clinicopathological features including germinal center immunophenotype status. The extent of each gene methylation status on patient's overall survival (OS) and disease-free survival (DFS) was assessed using the Kaplan-Meier method and compared with the log-rank test.
Results: Hypermethylation of SHP1 was associated with elevated lactate dehydrogenase level ($p = 0.031$). P16 and VHL were frequently hypermethylated in patients with high International Prognosis Index (IPI) scores ($p = 0.006$ and 0.004). In addition, hypermethylation of P16 was significantly associated with advanced clinical stages ($p = 0.041$). Interestingly, hypermethylation of DAPK was significantly correlated with resistance to treatment ($p = 0.023$). With regard to survival rates, promoter hypermethylation of DAPK, P16, and VHL were significantly associated with shortened OS ($p = 0.003$, 0.001, and 0.017, respectively) and DFS ($p = 0.006$, 0.003, and 0.046, respectively). In multivariate analysis, hypermethylation of DAPK remain an independent prognostic factor in predicting shortened OS ($p = 0.001$) and DFS ($p = 0.024$), as well as the IPI and the germinal center status.
Conclusions: In summary, our study demonstrates that DLBCLs with hypermethylated P16, VHL, DAPK, and SHP1 commonly show a biologically aggressive phenotype and worse prognosis. Interestingly, hypermethylation of DAPK was found to be a new independent prognostic factor that may be used to predict resistance to treatment and shortened survival in conjunction with the conventional prognostic factors such as the IPI and the germinal center status. Also, our results suggest that promoter gene methylation plays an important role in the pathogenesis of DLBCLs and may be a potential interesting target for determining new appropriate treatments.